theories and technologies that will be needed to bring these ideas to fruition. The tasks are challenging, even formidable, and require the commitment of scientists and engineers as well as the provision of vast funds for research, development and capital investment by governments and industry.

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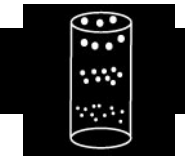
CATALYST STUDIES: CHROMATOGRAPHY



Isolation: Magnetic Techniques

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The first experiments with magnetic separation of cells date from the 1950s when lymphocytes were magnetically separated after *in vitro* phagocytosis of iron granules. The real boom in the application of magnetic labels for cell isolation came in the 1980s and since then an enormous amount of work has been done in both the development and application of this technique.

Magnetic separation of cells has several advantages in comparison with other techniques. In general, the magnetic separation procedure is gentle, facilitating the rapid handling of delicate cells in an unfriendly environment. It permits the cells of interest to be isolated directly from crude samples such as blood, bone marrow, tissue homogenates, stools, food, cultivation media, soil and water. The cells isolated by magnetic separation are usually pure, viable and unaltered. Magnetic separation is a simple, fast and efficient procedure and the whole separation process can be performed in the same tube. Large differences between magnetic permeabilities of the magnetic and nonmagnetic materials can be exploited in developing highly selective separation methods. The separation procedure can easily be scaled up if large quantities of living cells are required.

Principles of Magnetic Separation of Cells

Two types of magnetic separation can be distinguished when working with cells. In the first type, cells to be separated demonstrate sufficient intrinsic magnetic moment so that magnetic separations can be performed without any modification. There are only two types of such cells in nature: magnetotactic bacteria containing small magnetic particles within their cells and red blood cells (erythrocytes) containing high concentrations of paramagnetic haemoglobin. In the second type, cells of interest have to be tagged by a magnetic label to achieve the required contrast in magnetic susceptibility between the labelled and unlabelled cells. The attachment of magnetic labels is usually attained by the use of affinity ligands of various types, which can interact with target structures on the cell surface. Usually antibodies

against specific cells surface epitopes are used, but other specific ligands can also be employed. The newly formed complexes have magnetic properties and can be manipulated using an appropriate magnetic separator.

The magnetic separation process for the purification of target cells usually consists of the following three fundamentals steps:

1. The suspension containing cells of interest is mixed with magnetic labels. Incubation time is usually not longer than 30–60 min. Then the magnetic complex formed is separated using a magnetic separator and the supernatant is discarded or used for another application.
2. The magnetic complex is washed several times to remove unwanted contaminants. The selected cells with attached magnetic labels can be used directly, e.g. for cultivation experiments. Alternatively, cells can be disrupted and the cell content analysed using various methods.
3. If necessary, a variety of detachment procedures can be used to remove the magnetic labels from the separated cells. After detachment, the magnetic label is removed from the suspension in a separator and free cells are ready for further applications and analyses.

Necessary Equipment

Magnetic labels and magnetic separators are necessary to be able to perform efficient cell separation. Typical examples are given below.

Magnetic Labels

With the exception of erythrocytes and magnetotactic bacteria, the cells to be isolated have to be magnetically labelled in order to be amenable to magnetic treatment. Magnetic labelling can be performed with magnetic and superparamagnetic particles (*c.* 1 μm and more in diameter), magnetic colloids (*c.* 50–200 nm), magnetoliposomes or with molecular magnetic labels. In most cases the magnetic properties of the labels are caused by the presence of small particles of magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3); in some cases, chromium dioxide particles or ferrite particles have been used.

Magnetic and superparamagnetic particles Magnetic and superparamagnetic particles (typical diameter *c.* 1–5 μm) attached to the target cells can easily be removed from a suspension with a simple magnetic separator. Since there is usually no magnetic remanence the particles are not attracted to each other and therefore they can be easily suspended into a homogeneous mixture in the absence of any external magnetic field. Magnetic particles typically comprise fine grains of iron oxides dispersed throughout the interior of a polymer particle (in many cases of a monosized type), the surface chemistry of which can be modified to provide a range of different linking methods. Alternatively, silanized particles of magnetic iron oxides or magnetic porous glass can be used for the same purpose.

A number of particulate magnetic labels can be purchased commercially. Up to now, in most applications, monosized polymer particles marketed as Dynabeads (Dynal, Oslo, Norway) in various forms have been used. Dynabeads are prepared from monosized macroporous polystyrene particles which are magnetized by an *in situ* formation of ferromagnetic material inside the pores. Other commercially available magnetic particles can be successfully used for cell separation. A selection of these products can be found in **Table 1**.

Colloidal magnetic labels Colloidal magnetic labels (typical size *c.* 50–200 nm) are prepared by a variety of methods which result in flocks composed of polymer (typically dextran, starch or protein) and magnetite and/or other iron oxide crystals. A standard procedure for the synthesis of superparamagnetic dextran nanospheres is performed by precipitation of iron oxide in the presence of the polysaccharide. To such materials, ligands (usually antibodies, lectins, streptavidin or biotin) are coupled so they can be used for cell separation. Using high gradient magnetic columns, the labelled cells are easily separated. Magnetic particles isolated from magnetotactic bacteria (50–100 nm) composed of magnetite covered by a stable lipid membrane can also be used successfully.

Magnetoliposomes Magnetoliposomes are magnetic derivatives of ordinary liposomes prepared by incorporation of colloidal magnetic particles into the lipid vesicles. When magnetoliposomes are associated with antibodies they can label and/or selectively concentrate the target cells.

Molecular magnetic labels Molecular magnetic labels are usually lanthanides (especially erbium), ferritin and magnetoferritin. Erbium in the form of erbium chloride ($ErCl_3$) has been used for magnetic labelling of a variety of cells. Different Er^{3+} binding sites, such as carboxyl groups in glycoproteins or the Ca^{2+} receptor sites on the cell wall are responsible for the binding of erbium ions.

Table 1 Selected examples of commercially available magnetic particles and colloidal magnetic labels used or usable for magnetic separation of cells

Name	*Diameter* (μm)	*Polymer composition/surface modification*	*End groups and activation possibility*	*Immobilized compounds*	*Manufacturer/supplier*
BioMag	~ 1	Silanized iron oxides	–COOH –NH_2	Secondary Abs, anti-CD Abs, anti-fluorescein Ab, protein A, protein G, streptavidin, biotin	Perseptive Biosystems, Framingham, MA, USA
Dynabeads M-280 Dynabeads M-450 Dynabeads M-500	2.8 4.5 5	Polystyrene	Tosyl-activated	Secondary Abs, anti-CD Abs, Abs against *Escherichia coli* O157, *Salmonella*, *Listeria*, *Cryptosporidium*, streptavidin, oligo(dT)	Dynal, Oslo, Norway
Estapor	~ 1	Polystyrene	–COOH –NH_2		Prolabo, Fontenay-sous-Bois, France
Ferrofluids	0.135 0.175	Modified hydrophilic protein	–COOH –NH_2	Secondary Abs, protein A, streptavidin	Immunicon, Huntingdon Valley, PA, USA
M 100 M 104 M 108	1–10	Cellulose	–OH		Scigen, Sittingbourne, UK
MACS Microbeads	0.05	Dextran	–OH	Secondary Abs, anti-CD Abs, streptavidin, biotin	Miltenyi Biotec, Bergisch Gladbach, Germany
Magnetic microparticles	1–2	Polystyrene, cellulose, polyacrolein	–COOH –NH_2	Secondary Abs, protein A, protein G, streptavidin	Polysciences, Warrington, PA, USA
Magnetic nanoparticles	0.09–0.6	Starch, dextran, chitosan	–OH, –COOH	Streptavidin, biotin, protein A	Micro caps, Rostock, Germany
MagNIM	0.05 0.25 0.5		–COOH –NH_2	Secondary Abs, Ab against *E. coli* O157, streptavidin, protein A	Cardinal Associates, Santa Fe, NM, USA
Magnetic particles	~ 1	Polystyrene	–COOH –NH_2	Secondary Abs, Protein A, streptavidin,	Bangs Laboratories, Fishers, IN, USA
MPG	5	Porous glass	–NH_2, hydrazide, glyceryl	Streptavidin, avidin	CPG, Lincoln Park, NJ, USA
XM200 Microsphere	3.5	Polystyrene	–COOH	Secondary Abs, protein A	Advanced Biotechnologies, Epsom, UK

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Ferritin is a naturally occurring, soluble iron storage protein in mammals. For magnetic modification of cells cationized horse spleen ferritin (ferritin coupled with *N*,*N*-dimethyl-1,3-propanediamine) exhibiting a net positive charge at pH 7.5 is usually used. Under these conditions the cationized ferritin readily forms ionic bonds with the anionic sites on the cell membrane. Magnetic derivatives of ferritin called magnetoferritin, prepared by controlled reconstitution conditions, have also been used for cell labelling.

Magnetic Separators

A variety of magnetic separators is available on the market, starting with very simple concentrators for

one test tube and ending with complicated fully automated devices. In many cases, especially when working with larger labels, very cheap home-made magnetic separators can be used successfully.

Batch magnetic separators Batch magnetic separators are usually made from strong rare-earth permanent magnets embedded in disinfectant-proof material. The racks are designed to hold various sizes and numbers of tubes. Some of the separators have a removable magnet plate to facilitate easy washing of magnetic particles (**Figure 1**). Test tube magnetic separators enable separation of magnetic particles from volumes ranging between about 5 μL and 50 mL. It is also possible to separate cells from the wells of standard microtitration plates. Magnetic complexes from larger volumes of suspensions (up to approximately 500–1000 mL) can be separated using flat magnetic separators. More sophisticated magnetic separators are available, e.g. those based on the quadrupole and hexapole magnetic configuration.

Flow-through magnetic separators Flow-through magnetic separators are characterized by the flow of the liquid and suspended cells through the separation system. These systems are usually more expensive and more complicated in comparison with batch separators, but for preliminary experiments simple devices can also be used.

Laboratory-scale high gradient magnetic separtors (HGMS; **Figure 2**) are composed of small columns loosely packed with fine magnetic-grade stainless steel wool which are placed between the poles of strong permanent magnets or electromagnets. Magnetically labelled cells are pumped through the column, labelled cells are retained on the steel wool, the field is removed and cells are retrieved by flow and usually by gentle vibration of the column. Automation of the separation process has led to the development of computer-controlled magnetic cell sorters, such as CliniMACS (**Figure 3**) and AutoMACS, both produced by Miltenyi Biotec, Germany.

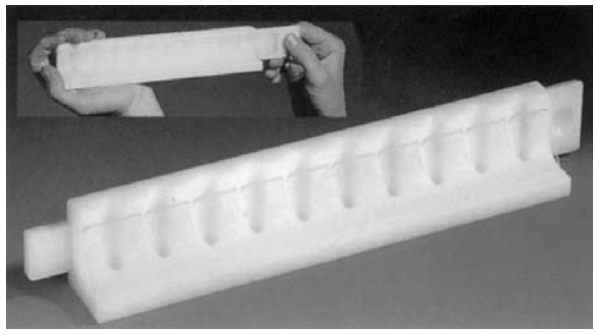

Figure 1 (See Colour Plate 63). Example of a magnetic separator (Dynal MPC-M) for work with microcentrifuge tubes of the Eppendorf type, with a removable magnet plate to facilitate easy washing of magnetic particles. Courtesy of Dynal, Oslo, Norway.

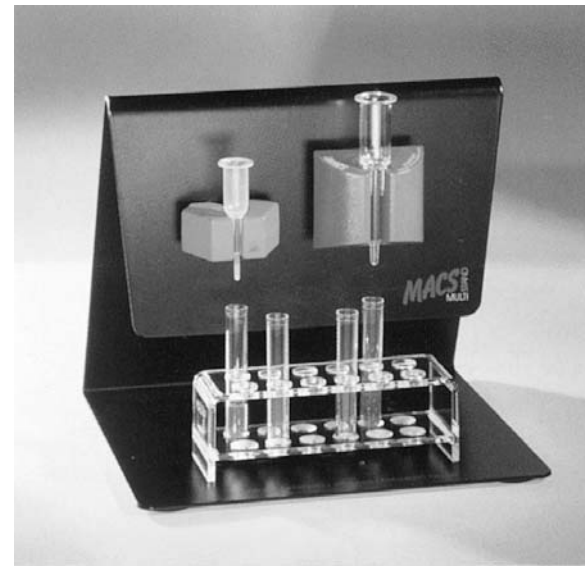

Figure 2 (See Colour Plate 64). Examples of laboratory-scale high gradient magnetic separators. Left: MiniMACS separation unit; right: MidiMACS separation unit, both with inserted columns. Courtesy of Miltenyi Biotec, Bergisch Gladbach, Germany.

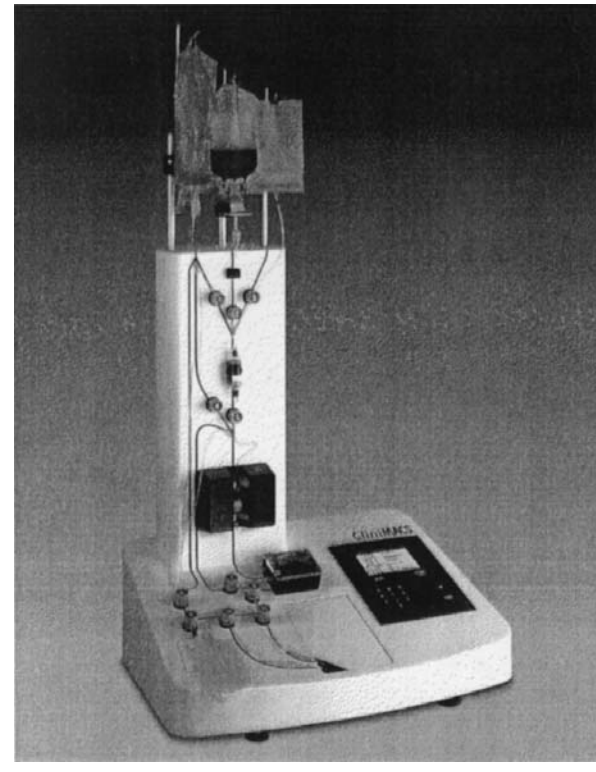

Figure 3 (See Colour Plate 65). Computer-controlled magnetic cell sorter CliniMACS. Courtesy of Miltenyi Biotec, Bergisch Gladbach, Germany.

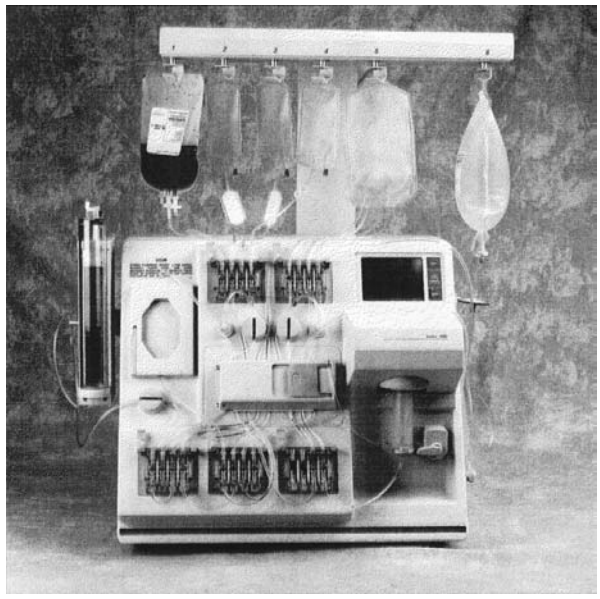

Figure 4 (See Colour Plate 66). The Isolex 300i Cell Selection System. Courtesy of Nexell Therapeutics, USA.

A continuous magnetic sorter based on an electrophoresis counter-flow chamber has been developed. The injected magnetic particles are deviated in the inhomogeneous magnetic field and focused into a stream that is completely separated from the streams of the undeviated particles.

The Isolex 300i Magnetic Cell Separator, produced by Nexell Therapeutics, USA, is intended for the isolation of stem cells from blood or bone marrow. The whole process is done automatically and the device represents a flexible platform for future applications in cell separation (**Figure 4**).

Procedures for Performing Magnetic Separation of Cells

Magnetic separation of cells is usually performed in one of the following formats:

1. Direct method. The affinity ligand is coupled to the magnetic particles, which are then added directly to the cell sample. During incubation the magnetic particles will bind the target cells which can be then recovered using a magnet.
2. Indirect method. Target cells are first sensitized with a suitable primary affinity ligand. After incubation, excess unbound affinity ligand is removed by washing the cells and then magnetic particles with an immobilized secondary affinity ligand with affinity for the first ligand are added. The magnetic particles will bind the target cells, which can then be recovered using a magnetic separator.

Another differentiation of magnetic separation techniques is based on the selection of the magnetically labelled cells.

1. Negative selection. Negative selection is a method by which a cellular subset is purified by removing all other cell types from the sample. Both the direct and indirect method are applied for negative selection. The advantage is that the purification process does not involve any direct contact of magnetic labels with the cells to be isolated.
2. Positive selection. The target cells are isolated from the cell suspension. Both the direct and indirect method can be used. The separated magnetically labelled cell complexes can be further characterized directly, but in many cases it is necessary to remove larger magnetic particles from the positively selected cells after their isolation.
3. Depletion of cells. Depletion is a method by which one or more unwanted cellular subsets is removed from a cell suspension. Both the direct and the indirect procedure can be applied for this purpose.

Immunomagnetic Separation

Immunomagnetic separation (IMS) is the most often used approach for the isolation of cells. Most often, a monoclonal antibody is used for IMS, but also polyclonal antibodies are used successfully. IMS can be performed in all the formats mentioned above.

In the direct method the appropriate antibody is coupled to the magnetic particles and colloids, which are then added directly to the sample. Ideally, the antibody should be oriented with its Fc part towards the magnetic particle so that the Fab region is pointing outwards from the particle. Several procedures are available for direct binding of antibodies (**Table 2**).

The indirect method is also used very often. The cell suspension is first incubated with primary antibodies which bind to the target cells. Not only purified primary antibodies have to be used, crude antibody preparations or serum can also be used. After incubation, the unbound antibodies are usually removed by washing. Then magnetic particles with immobilized secondary antibody are added to bind the labelled cells. Target cells – primary antibody complexes – can be also captured by protein A or protein G immobilized on magnetic carriers. Alternatively, biotinylated or fluorescein-labelled primary antibodies and magnetic particles with immobilized streptavidin or anti-fluorescein antibody are used to capture the target cells.

The indirect method is generally more efficient in removing target cells from a suspension because free antibodies will find their target antigen more easily

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Table 2 Selected procedures for binding of antibodies on magnetic particles and colloids

Adsorption of antibodies on hydrophobic magnetic particles (especially those made of polystyrene)
Covalent binding of antibodies on activated magnetic particles (e.g. tosyl-activated), or on magnetic particles carrying appropriate functional groups (e.g. carboxy, amino, hydroxy, hydrazide) using standard immobilization procedures
Immobilization of secondary antibodies (i.e. antibodies against primary antibodies) on magnetic particles followed by binding of primary antibodies
Immobilization of biotinylated antibodies on magnetic carriers with immobilized streptavidin
Immobilization of antibodies on magnetic particles with immobilized protein A and protein G
Immobilization of antibodies tagged with oligo dA on magnetic particles with immobilized oligo dT
Immobilization of antibodies on magnetic carriers with immobilized boronic acid derivative via their carbohydrate units on the Fc part

than antibodies bound to magnetic particles. The indirect technique is recommended when the target cell has a low surface antigen density or a cocktail of monoclonal antibodies is used. The direct method is usually faster and requires less antibody than the indirect method. Also, the direct method is advantageous when one does not want to cover all antigen sites with antibody.

Typically, 95–99% viability and purity of the positively isolated cells can be achieved with a typical yield of 60–99%. Depletion efficiency often reaches 99.9% and leaves remaining cells untouched. Sequential depletions are markedly more efficient.

Magnetic particles usually do not have any negative effect on the viability of the attached cells. Many types of magnetic particles are usually compatible with subsequent analytical techniques such as flow cytometry, electron and fluorescence microscopy, polymerase chain reaction (PCR), fluorescence *in situ* hybridization (FISH) or cultivation in appropriate nutrient media. In some cases, however, it is necessary to remove larger immunomagnetic particles from the cells after their isolation. The detachment process can be performed in several ways (**Table 3**).

Incubation time for cell separation is usually 5–60 min while the binding of primary antibodies to secondary coated magnetic particles usually takes 30 min or less. In positive isolation, the purity of cells generally decreases with time, although the yield increases.

Nonspecific interactions of nontarget cells with hydrophobic magnetic particles can be expected. These interactions can be partially eliminated using bovine or human serum albumin, casein and nonionic tensides such as Tween 20.

Magnetic Separations using other Labels

Antigens Antigens immobilized on magnetic particles can be used for the isolation of antibody expressing or antigen-specific cells. This approach has been successfully used for selection of antigen-specific hybridoma cells or human antibody-producing cell lines.

Lectins Lectins immobilized on magnetic carriers can interact with saccharide residues on the cell surfaces. A typical example of this approach is the application of immobilized *Ulex europaeus* I lectin which binds to terminal L-fucosyl residues present on the surface of human endothelial cells. Magnetic beads can be released from the isolated cells using a free competing sugar.

Oligosaccharides Oligosaccharides immobilized on magnetic particles can be used for the rapid isolation of specific lectin-expressing cells. Target cells bound to the magnetic particles can be released using a free competing saccharide structure.

Bacteriophage *Salmonella*-specific bacteriophage immobilized to a magnetic solid phase has been used for the separation and concentration of *Salmonella* from food materials.

Table 3 Selected typical procedures for detachment of cells after immunomagnetic separation

Incubation of rosetted cells overnight in cell culture medium with subsequent mechanical forces such as firm pipetting, flushing the suspension 5–10 times through a narrow-tipped pipette
Trypsin, chymotrypsin and pronase have general applicability for proteolytic detachment of isolated cells
Detachment with a polyclonal antibody that reacts with the Fab fragments of primary monoclonal antibodies on magnetic beads. This principle is commercialized by Dynal, Norway (DETACHaBEAD)
Using synthetic peptides which bind specifically to the antigen-binding site of primary antibodies (Baxter Healthcare, Deerfield, IL, USA)
Antibodies immobilized via carbohydrate units on the Fc part to the magnetic particles with immobilized $-B(OH)_3$ groups are dissociated with sorbitol
A complex primary antibody–DNA linker can be split enzymatically using DNase
Cryptosporidium oocysts were successfully released from the immunomagnetic particles by decreasing the pH of the suspension (adding HCl)

Erbium ions, ferritin and magnetoferritin have been used for magnetic labelling of both prokaryotic and eukaryotic cells. Magnetotactic bacteria can be introduced into granulocytes and monocytes by phagocytosis which enables their magnetic separation. Submicron magnetic particles of γ-Fe_2O_3 adhere to the surface of *Saccharomyces cerevisiae*, making the cells magnetic and amenable to magnetic separation.

Magnetotactic bacteria, due to the presence of ferromagnetic material in their cells, can be magnetically separated without any labelling. Erythrocytes can be separated by the high gradient magnetic separation technique after conversion of diamagnetic erythrocytes containing oxyferrohaemoglobin into paramagnetic red blood cells by the oxidation of the iron atoms in the cell haemoglobin to the ferric state (methaemoglobin). Erythrocytes, infected by *Plasmodium*, contain paramagnetic hemozoin, that is a component of malarial pigment. The paramagnetic moment of hemozoin is of sufficient magnitude to enable the separation of malaria-infected (hemozoin-bearing) erythrocytes.

Magnetic Separations in Microbiology, Cell Biology, Medicine and Parasitology

IMS and, in some cases, lectin-magnetic separations are often used in the above-mentioned disciplines. In microbiology they are especially used for the detection of pathogenic microorganisms. IMS enables the time necessary for detection of the target pathogen to be shortened. Target cells are magnetically separated directly from the sample or the pre-enrichment medium. Isolated cells can than be identified by standard, specific microbiological procedures. IMS is not only faster but also usually gives a higher number of positive samples. Also sublethally injured and stressed microbial cells can be very efficiently isolated using IMS. The most important microbial pathogens can be detected using commercially available specific immunomagnetic particles; they are used for the detection of *Salmonella*, *Listeria* and *Escherichia coli* O157. New immunomagnetic particles for the detection of other microbial pathogens are under development.

Removal of cancer cells is one of the most important applications of IMS in the area of cell biology and medicine. The first experiments were performed in the 1970s and since then an enormous number of applications have been described. Cancer cells are usually removed from bone marrow prior to its autologous transplantation and using IMS they are detected in blood. Elimination of graft-versus-host disease (GvHD) in allogenic bone marrow transplantation requires an effective removal of T cells from the bone marrow of the donor. A direct method enabled a 10^3 times depletion of T cells.

Magnetic particles are being increasingly used for isolation of human cell subsets directly from blood and other cell sources. B lymphocytes, endothelial cells, granulocytes, haematopoietic progenitor cells, Langerhans cells, leukocytes, monocytes, natural killer cells, reticulocytes, T lymphocytes, spermatozoa and many others may serve as examples. Cells from other animal and plant species have been successfully separated, too.

Not only whole cells, but also cell organelles can be isolated from crude cellular fractions. Dynal (Oslo, Norway) has developed Dynabeads M-500 Subcellular, which are able to isolate rapidly more than 99% of target organelles.

In the area of parasitology *Cryptosporidium* and *Giardia* are the parasites where IMS is of interest. Two commercially available kits can be used for this purpose. Both products are used in the method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA (December 1997 Draft) of the US Environmental Protection Agency. In very low turbidity samples (clean waters), IMS has demonstrated significantly better results than the standard procedures. When water samples were turbid, the recovery efficiency of IMS diminished.

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Future Developments

Magnetic separation of cells is a simple, rapid, specific and relatively inexpensive procedure, which enables the target cells to be isolated directly from crude samples containing a large amount of nontarget cells or cell fragments. Many ready-to-use products are available and the basic equipment for standard work is relatively inexpensive. The separation process can be relatively easily scaled up and thus large amount of cells can be isolated. New processes for detachment of larger magnetic particles from isolated cells enable use of free cells for *in vivo* applications. Modern instrumentation is available on the market, enabling all the process to run automatically. Such devices represent a flexible platform for future applications in cell separation.

IMS play a dominant role at present but other specific affinity ligands such as lectins, carbohydrates or antigens will probably be used more often in the near future. There are also many possibilities to combine the process of cell magnetic separation with other techniques, such as PCR, enabling the elimination of compounds possibly inhibiting DNA polymerase. New applications can be expected, especially in microbiology (isolation and detection of

microbial pathogens) and parasitology (isolation and detection of protozoan parasites). No doubt many new processes and applications in other fields of biosciences and biotechnologies will be developed in the near future.

See Colour Plates 63, 64, 65, 66.

See also: **II/Centrifugation:** Analytical Centrifugation; Large-Scale Centrifugation. **III / Cells and Cell Organelles:** Field Flow Fractionation.

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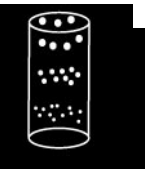
CELLS AND CELL ORGANELLES: FIELD FLOW FRACTIONATION



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Introduction

Analysis and sorting of living cells and purification of cell organelles are important procedures in the life sciences. There is a wide range of techniques and methodologies available, which can be divided into three main groups. The techniques in the first groups are based on physical criteria such as species size, density and shape, and include centrifugation, elutriation and field flow fractionation. Those in the second group are linked to cell surface characteristics, while flow cytometry techniques make up the third group. At a fundamental level, field flow fractionation (FFF) exploits the physical characteristics of the cells or cell organelles. However, cells or cell organelles exhibit some specific characteristics that can be described by a multipolydispersity matrix. The different physical characteristics of these biological materials require different FFF techniques and modes of operation. Special care must be taken if biological integrity and viability are to be preserved.

Specific Cell Characteristics

Cellular materials range in size from 1 μm to 50 μm. Cell populations are classified by a set of morphological, functional and biophysical characteristics. The biophysical characteristics are of particular interest in FFF. Usually, separations in FFF are influenced (but not directed) by surface properties of the sample components (to avoid particle–particle or particle–separator interactions). These properties can be modulated by the use of appropriate carrier-phase modifiers (surfactants). In terms of FFF separations, mass, size and density appear to be the major first order parameters. However, size is generally defined by the radius or the diameter of a sphere whose volume is identical to that of the cell. Size can therefore be deduced accurately if the cell of interest is perfectly spherical. However, this is not usually the case, and the sphericity index, I, is then used:

$$I = \frac{4.84(V^{2/3})}{S}$$

In this equation V is the cell volume and S is its surface area can be difficult to determine. In terms of cell population, these dimensions are averages and should be associated with a variance. These general